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Response of the central metabolism of *Escherichia coli* to modified expression of the gene encoding the glucose-6-phosphate dehydrogenase

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Abstract The deletion of the *zwf* gene encoding G6PDH activity led to restructuring of the carbon flux through central metabolism in *Escherichia coli*, though over-expression of this gene had only minor consequences for overall carbon flux. The modified carbon flux seen in the *zwf* deletion mutant enabled alternative routes of anabolic precursor formation and an adequate supply of NADPH synthesis via a modified TCA cycle to be generated so as to sustain growth rates comparable to the WT.

Keywords: Metabolic network; Fluxomics; ^{13}C-labelling experiments; Mass isotopomer analysis; *Escherichia coli*

1. Introduction

Microorganisms can efficiently adapt their metabolism in response to genetic or environmental changes, and understanding the extent of this metabolic robustness has become an emergent issue in both basic and applied research. Part of the robustness is directly related to the network organization of metabolic systems, where the interplay between all available biochemical reactions provides alternative mechanisms for compensating the perturbations [1,2]. Recently, ^{13}C-metabolic flux analysis (^{13}C-MFA) has been applied to *Escherichia coli* knock-out mutants lacking key enzymes of central metabolism to determine the phenotypic effects of structural changes in the metabolic network, providing direct evidence for the nature and extent of the mechanisms that compensate the effects of such perturbations [3–6]. Compensatory mechanisms involve enzyme redundancy and alternative pathways.

Glucose-6-phosphate dehydrogenase (G6PDH) is a key enzyme in central metabolism, involved in the partition of carbon between glycolysis and the pentose phosphate pathway (PPP) which provides a large proportion of the NADPH needed for anabolism. The consequences of the lack of G6PDH have been already reported [7–9], but there is currently no report on the effects of increased G6PDH activity on the metabolic network. A strain expressing *zwf* to high levels has been constructed and compared to both its parent strain and an isogenic strain in which *zwf* has been deleted as regards to the growth characteristics and metabolic flux distribution. To define clearly the consequences of specific genetic modifications, isotopic flux distribution techniques were used to access real in vivo metabolic fluxes within central metabolism.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli MG1655 was used as the parent strain for the construction of both the deletion and overexpression mutants. The deletion mutant (Δzwf) was obtained by a one-step disruption protocol [10] and the overexpression mutant (Pzwf) was obtained by a one-step method to modulate expression of chromosomal genes [11] based on sequence substitution within the promoter region. In order to confirm the mutations, polymerase chain reaction (PCR) was used to amplify fragments containing the modified sequences. Lengths of amplified fragments were tested by agarose gel electrophoresis and compared with those of the wild type strain (WT). PCR products were also sequenced to confirm *zwf* removal and to determine the exact sequence of the artificial promoter.

2.2. Media and growth conditions

All *E. coli* strains were grown on minimal synthetic medium containing 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1 g/l of thiamine and 2 g/l of glucose. Magnesium sulfate and calcium chloride were autoclaved separately. Glucose and thiamine were sterilized by filtration. Exponentially growing cells pre-cultured in the same medium were harvested by centrifugation, washed in the same volume of fresh medium (lacking glucose and thiamine) and used for inoculation with 1% (v/v). Cultivation

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Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; ^{13}C-MFA, ^{13}C-metabolic flux analysis; GC–MS, gas chromatography–mass spectrometry; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle; ED, Entner Doudoroff

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was at 37 °C in a 2 l stirred reactor with a dissolved oxygen content maintained above 20% saturation and with pH regulated at 6.9 by addition of 2 M NaOH.

2.3. Analytical procedures and physiological parameters

Cell growth was monitored by measuring optical density at 600 nm (OD_{600}). Glucose and acetate concentrations were determined by HPLC as described previously [12]. Physiological parameters (maximum growth, specific glucose consumption and specific acetate production rates) were determined during exponential growth phase using a previously validated correlation factor of 0.38 g cellular dry weight per OD_{600} U.

The specific activity of G6PDH was measured in crude cell extracts. After two washings, the cells were resuspended in buffer containing Tris–HCl (275 mM) and tricarballylate (10 mM) at pH 7.8, glycerol (10% v/v), $MgCl_2$ (11 mM) and DTT (1 mM). Cells were disrupted by sonication using an ultrasonic disruptor (Bioblock Vibracell 72412). Cell debris was removed by centrifugation (20 000 × *g* for 10 min at 4 °C), and the crude cell extracts were used for enzyme assays. Enzyme activity was measured spectrophotometrically at 340 nm under thermostatically controlled conditions (37 °C). All compounds of the reaction mixture, Tris–HCl buffer pH 7.6 (100 mM), $MgCl_2$ (12.5 mM), $NADP^+$ (0.6 mM), 350 µl extract, diluted as necessary, were pipetted into a micro-cuvette with a 1 cm light path. The reaction was initiated by adding glucose 6-phosphate (2 mM) to a final volume of 1 ml. Protein concentrations were determined using the Lowry method with bovine serum albumin as a standard [13].

2.4. ^{13}C-labelling experiments

Cultures for ^{13}C-labelling experiments employed a mixture of 20% (mol/mol) [U-^{13}C] glucose and 80% (mol/mol) [1-^{13}C] glucose (99% of ^{13}C atom, Eurisotop, France). Samples were collected at the mid-exponential phase to ensure isotopic and metabolic steady state. Mass isotopomer fractions of amino acids were determined in triplicate by gas chromatography–mass spectroscopy (GC–MS) as described previously [14]. Data acquisition were performed in the selected ion monitoring (SIM) mode, monitoring mainly fragments at $[M-57]^+$ except for isoleucine. The mean experimental error for mass isotopomer fractions was 0.38%. The GC–MS derived mass isotope distributions were corrected for naturally occurring isotopes [15]. The measurement of positional isotopomer and carbon enrichments was performed by NMR spectroscopy, from 2D-HSQC [16] and 2D-TOCSY [17] experiments, respectively. Samples for NMR analysis were prepared as described previously [17] from the same cultures as the samples used for GC–MS analysis.

2.5. Metabolic flux analysis

Metabolic fluxes were calculated from the ^{13}C-labeling patterns of metabolites using both metabolite and isotope balancing equations. The metabolic network considered for flux calculations contained the main pathways of *E. coli* central metabolism: glycolysis, PPP, tricarboxylic acid cycle (TCA), the glyoxylate shunt and anaplerotic reactions. It included additionally the Entner Doudoroff (ED) pathway and reactions of amino acid biosynthesis. The reaction between phosphoenolpyruvate and oxaloacetate was defined as the net flux through a bidirectional conversion involving phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase.

Flux calculations were performed using the 13C-Flux software developed by Wiechert [18], in which both mass balances and carbon atom transitions describing the bioreaction network were used. Measurable extracellular fluxes (glucose uptake, acetate production and specific precursor requirements for biomass [19]) were constrained as well as the labelling pattern of glucose, thereby obtaining direct evidence for possible modifications in in vivo fluxes.

3. Results and discussion

3.1. Construction and characterization of the mutants

The behaviour of the strain over-expressing *zwf* (Pzwf strain) (in which the promoter sequence was modified as shown in Supplementary data S1) was compared to its parent strain (*E. coli* MG1655) but also to a knock-out mutant lacking G6PDH activity (Δzwf strain), generated by removal of the entire sequence of the *zwf* gene.

The expression of *zwf* in these strains was assessed by measuring G6PDH activity in cell-free extracts. The $NADP^+$-dependent G6PDH activity of the WT strain was found to be 190 ± 4 nmol/min/$mg_{protein}$. This value was similar to the values reported in the literature for comparable cultivation conditions [4,5]. As expected, no G6PDH activity was detectable in the deletion mutant while high activity was measured in the over-expression mutant (2870 ± 20 nmol/min/$mg_{protein}$). This value was more than 15 times higher than in the parent strain, indicating that the *zwf* gene was efficiently over-expressed when placed under the control of the artificial promoter.

The growth behaviour of the strain over-expressing *zwf*was compared to the growth of the two other strains (Table 1). The growth of the parent strain in the medium considered in this work was similar to that seen in previous reports using synthetic media, and was accompanied by the production of acetate. As described previously [9], the deletion of the *zwf* gene had little effect on cell growth (slight decrease in growth rate). In the same way, the strain over-expressing *zwf* showed growth characteristics close to those observed for the WT strain, indicating that the increased G6PDH activity had no significant consequence on the overall phenotypic behaviour. Obviously, the metabolic network of *E. coli* can compensate for such changes in *zwf* expression without major deleterious effects on the growth characteristics.

3.2. Metabolic flux response to modified expression of zwf

The distribution of carbon flux throughout the central metabolic pathways was measured in the three strains, using ^{13}C-labelling experiments. Flux analysis was based on the GC–MS mass isotopomer analysis of proteinogenic amino acids following proteolysis of biomass collected under both metabolic and isotopic steady-state conditions [20]. For this purpose, cells were grown in minimal medium containing a mixture of 80% [1-^{13}C] glucose and 20% [U-^{13}C] glucose. The specifically labelled [1-^{13}C] glucose was used, in addition to the uniformly labelled glucose, to obtain direct data concerning flux through the oxidative PPP since CO_2 loss within this pathway involves specific loss of [1-^{13}C] glucose [21]. Thus, the M0 abundances will reflect the extent to which glucose is catabolized via PPP. The M0 abundances, corresponding to unlabelled molecules, for all amino acids were lower in the *zwf* deletion mutant than in the two other strains (see Supplementary Table S2). The higher content in ^{13}C-atom in this strain thus reflects a diminished contribution of the oxidative PPP in this strain. The relative importance of M0 abundances was quite similar in the WT and the strain over-expressing *zwf*, indicating that flux distribution was not significantly altered in this background.

Table 1
Growth characteristics of the different strains: μ, specific growth rate; q_{Glc}, glucose uptake rate; v_{Ac}m acetate production rate

Strains	μ (h^{-1})	q_{Glc} (mmol/g/h)	v_{Ac} (mmol/g/h)
Δzwf	0.60 ± 0.04	7.71 ± 0.43	3.40 ± 0.19
WT	0.65 ± 0.01	7.15 ± 0.61	3.44 ± 0.42
Pzwf	0.66 ± 0.02	7.32 ± 0.65	3.23 ± 0.29

The GC–MS data and measured extracellular fluxes were used to measure the flux distributions within the central metabolic pathways of *E. coli* using established fitting procedures [16]. The flux distributions for the three strains are mapped in Fig. 1A, in which net fluxes are expressed relative to the glucose uptake rate. The correlation (linear regression coefficients close to 0.998) between the experimental and the calculated labeling data were high for all three strains (Fig. 1B) indicating that the metabolic model used to resolve the flux distributions was complete and did not introduce any data handling artifacts.

The G6PDH catalyses the first step of the PPP, a pathway that can serve as a catabolic route but also provides both carbon precursors (ribose-5-phosphate and erythrose-4-phosphate) and reductive power (i.e. NADPH) for anabolic purpose. Alterations of this step might therefore be expected to have significant effects on the metabolic behaviour of

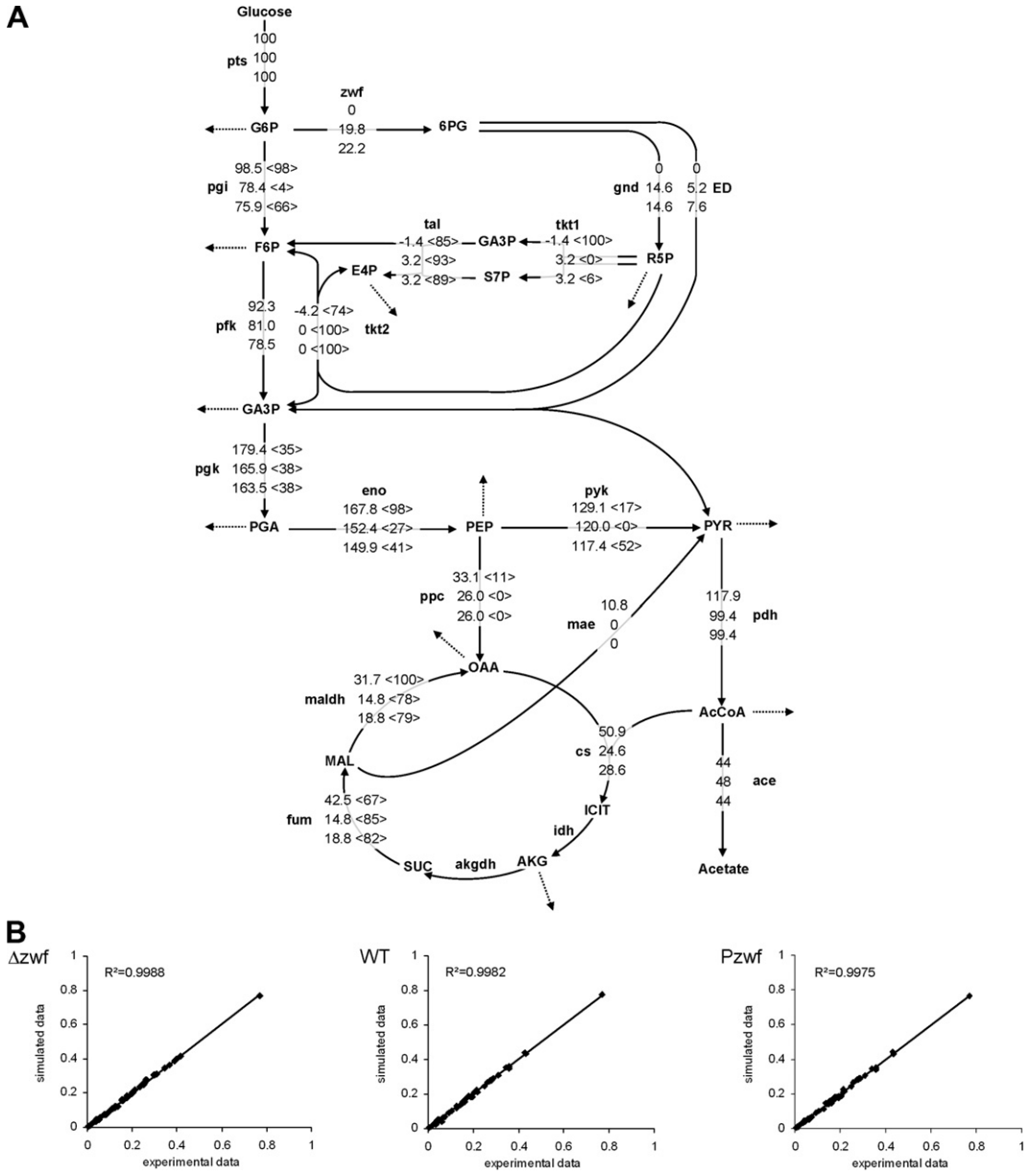

Fig. 1. (A) Flux distributions within central metabolism for three strains of *E. coli*: upper values, *zwf* deletion mutant; middle values, WT strain; lower values, strain over-expressing *zwf*. The reversibility of the reactions is expressed in percentage of the forward flux and is shown in brackets. pts, PTS glucose transport; pgi, phosphoglucoisomerase; pfk, phosphofructokinase + aldolase; pgk, glyceraldehyde-3-phosphate dehydrogenase + phosphoglycerate kinase/mutase; eno, enolase; pyk, pyruvate kinase; ppc/pck, PEP carboxylase/PEP carboxykinase; mae, malic enzyme; pdh, pyruvate dehydrogenase; ace, acetate production rate; cs, citrate synthase; idh, aconitase + isocitrate dehydrogenase; akgdh, 2-oxoglutarate dehydrogenase + succinyl-CoA synthetase; fum, succinate dehydrogenase + fumarase; maldh, malate dehydrogenase; gnd, 6-phosphogluconate dehydrogenase + isomerase/epimerase; tkt1, transketolase 1; tal, transaldolase; tkt2, transketolase 2; ED, ED pathway. Dashed arrows represent fluxes towards biomass synthesis. The corresponding specific glucose uptake rates were rather similar for the different strains (Table 1) so that the strain specific differences shown for the relative fluxes also hold in terms of absolute carbon flux. (B) Correlations between GC–MS experimental data and simulated data.

E. coli. The distribution of carbon fluxes in the *zwf* deletion mutant showed that the anabolic requirements for erythrose-4-phosphate and ribose-5-phosphate were satisfied by the reverse activity of the non-oxidative branch of the PPP. The ^{13}C-isotope flux data indicates that NADPH synthesis was compensated by an increased flux through the TCA cycle and probably via a flux through malic enzyme. However, the inherent difficulties in resolving anaplerotic fluxes using GC–MS techniques are such that this probable participation could not be conclusively established. In order to confirm the flux through this alternative pathway, NMR analysis giving increased precision over these reactions was undertaken (see NMR data in Supplementary Table S3), clearly confirming the existence of this flux through malic enzyme (Fig. 2). Thus, carbon flux entering the TCA cycle was increased twofold and a significant part of this flux was oriented out of the TCA cycle via malic enzyme with a corresponding increase in OAA replenishment via PEP carboxylase. This alternative pathway for NADPH synthesis has been proposed for other microorganisms, e.g. [22] though clear experimental evidence to support this hypothetical pathway was until now lacking. Analysis of the literature for *zwf* mutants of *E. coli* [8,9,23] does not provide an obvious indication of this function. While certain authors have measured a flux through malic enzyme this flux was either not specific to the *zwf* deletion mutation or accompanied by a diminished flux in PEP carboxylase. The flux analysis obtained in the present study, clearly illustrates that this pathway functions and contributes to NADPH-generation in the *zwf* deletion mutant, albeit to a rather limited extent (approximately 6% of total NADPH requirements for growth). The consequence of this restructuring of metabolic fluxes is a virtually unchanged rate of NADPH synthesis as compared with the WT and the strain over-expressing *zwf* (Table 2) though in all cases NADPH supply was insufficient to meet calculated anabolic requirements, confirming previous suggestions that transhydrogenase reactions contribute to NADPH supply [20]. However, this reaction cannot apparently substitute for the NADPH supply normally derived from the oxidative branch of the PPP despite a significantly increased overproduction of other cofactors (ATP, NADH and FADH) in the *zwf* deletion mutant (see Table 2). Since the biomass yield in this strain is slightly lower than in the two other strains, despite energy excess condition, the modified flux distribution is either accompanied by an enhanced level of energy wastage involving mechanisms whose nature has yet to be identified or rate-limitations in the rate of anabolic precursor supply presumably in the modified configuration of PPP. It is interesting to note, that in all the genetic backgrounds acetate production remained constant.

A pathway for PEP oxidation alternative to the TCA cycle was recently proposed from ^{13}C-labelling experiments in starving *E. coli* cells [24]. Upon strong glucose limitation, PEP was fully oxidized to CO_2 by an NADP-independent pathway involving the glyoxylate shunt and PEP carboxykinase. This process decouples catabolism from NADPH formation in conditions of slowed growth. The process of PEP oxidation ob-

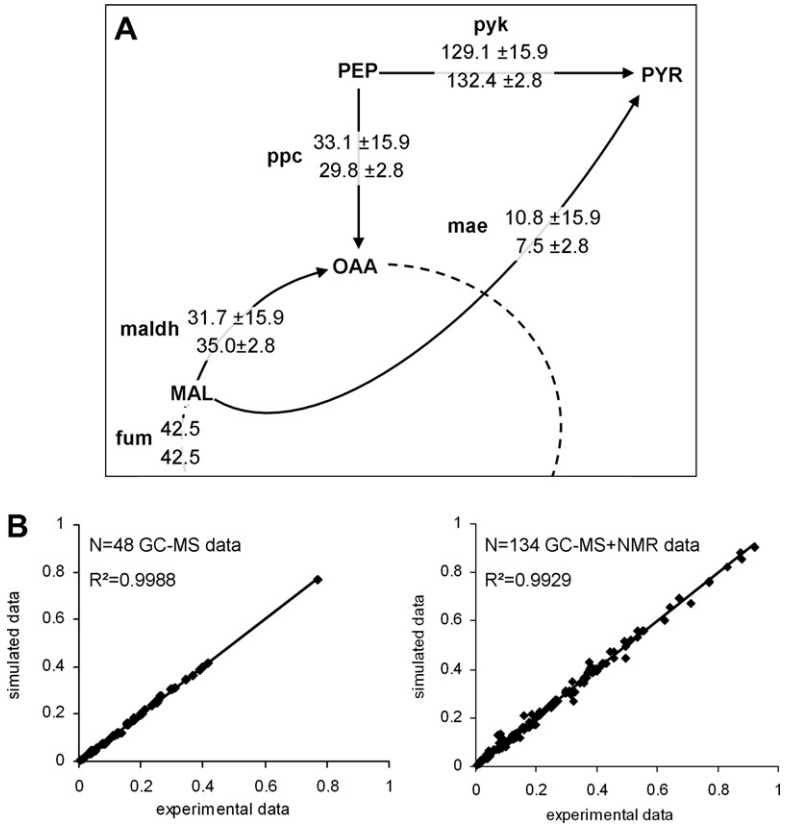

Fig. 2. Distribution of anaplerotic fluxes in *zwf* deletion mutant. (A) Flux distributions obtained from GC–MS data (upper values), or from the combination of GC–MS + NMR data (lower values). (B) Correlations between experimental data and simulated data for flux simulation carried out with either GC–MS data (left) or with GC–MS + NMR data (right). The total number of isotopic data considered in each simulation is indicated.

Table 2
Rates (relative to 100 $mmol_{glc}$/g/h) of cofactor supply and anabolic requirements based on the growth kinetics and metabolic flux distributions in the *E. coli* strains having different levels of *zwf* expression

Strains	XADH[a] supply	NADPH requirements	NADPH via PPP	NADPH via Idh	NADPH via Mae	NADPH via TH[b]	ATP requirements	ATP supply
Δzwf[c]	468	128	0	50.9	7.5	70	308	206
WT	343	150	34.4	24.6	0	91	360	168
Pzwf	352	149	36.8	28.6	0	84	357	165

[a]NADH and FADH.
[b]TH: transhydrogenase.
[c]Calculated using the flux data obtained via NMR analysis.

served in the present work was different in terms of metabolic sequence, cofactor formation and conditions of expression, and is consistent with situations of insufficient NADPH formation. However, it also relies on the activation of anaplerotic reactions to generate an alternative pathway for complete oxidation of carbohydrates. It is a further illustration of the flexibility of *E. coli* metabolism around the TCA cycle and emphasizes the central role of anaplerosis in metabolic adaptation [25].

Flux distribution in the *zwf*-over-expressing mutant was similar to that obtained for the WT parent strain (Fig. 1A). The flux through the G6PDH reaction was only slightly higher in the strain over-expressing *zwf* than in the WT strain but this increased flux was entirely directed towards the ED pathway. Since, the 15-fold increase in G6PDH activity measured in vitro in this mutant did not provoke a significant increase through the corresponding reaction, it may be concluded that this flux is tightly controlled either by biochemical regulation of G6PDH activity or indirectly via reactions downstream of G6PDH in the PPP.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.06.066.

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